JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2005, p. 6120–6122 Vol. 43, No. 12
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.12.6120–6122.2005

Frequency and Species Distribution of Gliotoxin-Producing *Aspergillus* Isolates Recovered from Patients at a Tertiary-Care Cancer Center

Russell E. Lewis,[1,2]* Nathan P. Wiederhold,[3] Michail S. Lionakis,[2] Randall A. Prince,[1,2] and Dimitrios P. Kontoyiannis[1,2]

The University of Houston College of Pharmacy, Houston, Texas[1]; The University of Texas M.D. Anderson Cancer Center, Houston, Texas[2]; and The University of Texas at Austin College of Pharmacy, Austin, Texas[3]

Received 23 July 2005/Returned for modification 13 September 2005/Accepted 4 October 2005

***Aspergillus* isolates ($n = 103$) collected from cancer patients were screened to determine the taxonomic distribution and quantity of gliotoxin production. Gliotoxin was detected in 93% of *Aspergillus fumigatus*, 75% of *A. niger*, 25% of *A. terreus*, and 4% of *A. flavus* cultures. Gliotoxin concentrations were highest in cultures of *A. fumigatus*.**

Aspergillus fumigatus produces several secondary metabolites during invasive hyphal growth (4, 12). One of the most abundantly produced metabolites is the epipolythiodioxopiperazine metabolite gliotoxin, which exhibits a diverse array of biologic effects on the immune system. Gliotoxin inhibits macrophage and polymorphonuclear cell function and the generation of alloreactive cytotoxic T cells (2, 3, 7, 13, 14) and suppresses activation of the nuclear transcription factor NFκB—the central regulator of gene transcription for inflammatory cytokines, growth factor receptors, and cell adhesion molecules (9). In immunocompetent mice, the administration of gliotoxin results in a level of immunosuppression sufficient to render animals susceptible to fatal invasive aspergillosis after challenge with *A. fumigatus* conidia (11). These immunosuppressive properties, in conjunction with the release of the mycotoxin by the invasive hyphal form, suggest that gliotoxin production in situ could aid the evasion of fungal hyphae from professional effector cells of the host immune response and contribute to the pathobiology of invasive aspergillosis (12).

Little is known about the biosynthesis or primary role of gliotoxin production in *Aspergillus* species. Interestingly, not all *Aspergillus* species appear to be capable of producing gliotoxin (3). Horizontal transmission of gene clusters involved in epipolythiodioxopiperazine synthesis has been proposed as a possible reason for the discontinuous evolution of mycotoxin production in fungi (3). Clearly, the ability to produce immunosuppressive secondary metabolites could have advantages for the growth and persistence of a saprophytic mold in humans. However, no published studies have examined the frequency and distribution of gliotoxin production among *Aspergillus* isolates recovered from patients at risk for invasive aspergillosis. To this end, we screened 103 consecutive *Aspergillus* isolates from cancer patients for the production of gliotoxin.

(This work was presented previously [R. E. Lewis, N. P. Wiederhold, M. S. Lionakis, J. Chi, R. A. Prince, and D. P. Kontoyiannis, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-245, 2004]).

Collection of clinical isolates and of corresponding patient data from electronic medical records was carried out in accordance with institution standards and with the approval of the internal review committees. Consecutive *Aspergillus* isolates ($n = 103$) recovered from respiratory and tissue specimens from 1998 to 2003 at The University of Texas M. D. Anderson Cancer Center Clinical Microbiology Laboratory were collected and stored using routine microbiological methods. All isolates were subcultured twice on potato dextrose agar slants (Remel, Lenexa, KS) and incubated at 37°C for 5 days. Conidia were then harvested from slants by flooding agar with 0.85% NaCl/0.2% Tween 80 and filtering the suspension through sterile gauze to remove hyphal fragments. The resulting conidium suspension was then adjusted by use of a spectrophotometer to 80% transmittance (1×10^6 to 2.5×10^6 conidia/ml) before 1:10 dilution in RPMI 1640 growth medium plus 0.165 M MOPS [2-(*N*-morpholino)propanesulfonic acid] plus 2% glucose (pH 7.0).

Gliotoxin concentrations were analyzed in culture media in duplicate by use of high-performance liquid chromatography (HPLC). Culture medium was centrifuged at $140,000 \times g$ for 4 min at 10°C. Supernatant (200 μl) plus an internal standard (3-nitrophenol) was then extracted by solid-phase extraction with a 10-kHz-cutoff filter system (Sep-Pak; Waters, Milford, MA). The elution (80 μl) was then injected in the HPLC system, which consisted of a Waters 2487 dual λ absorbance detector with a wavelength set to 270 nm and a 4.6- by 250-mm Alltech Altima C_{18} reverse column packed with 5-μm particles. The mobile phase consisted of acetonitrile and water (43:57 volume/volume) run at a flow rate of 1 ml/min. Gliotoxin concentrations were determined by interpolation from a 7-point standard curve (25 ng/ml to 1,000 ng/ml) prepared using a purified gliotoxin powder (Sigma, St. Louis, Mo.). Overall recovery of gliotoxin from culture media exceeded 90%, and both inter- and intra-assay coefficients of variation were less than 10% over the range of the standard curve. A mock-inoculated sample consisting of gliotoxin (250 to 500 ng/ml) in RPMI culture medium was analyzed with each HPLC run as a

* Corresponding author. Mailing address: University of Houston College of Pharmacy, Texas Medical Center Campus, 1441 Moursund St. #423, Houston, TX 77030. Phone: (713) 795-8326. Fax: (713) 795-8383. E-mail: rlewis@uh.edu.

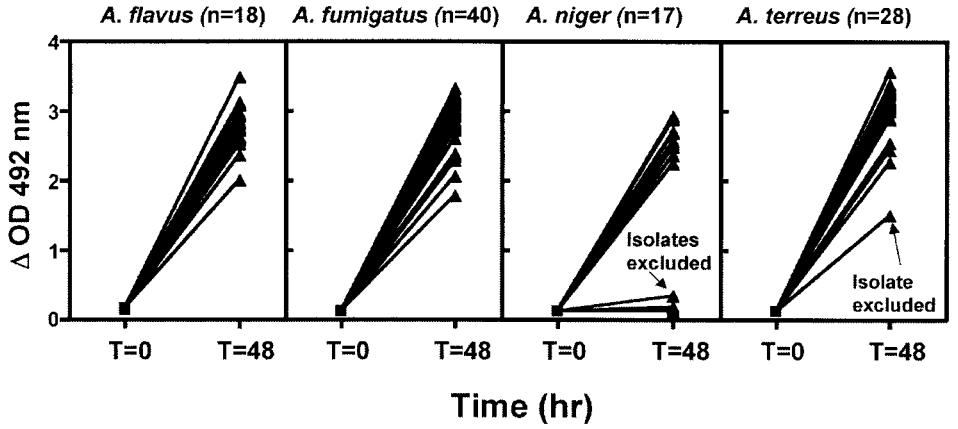

FIG. 1. Growth rates of 103 clinical *Aspergillus* isolates screened for production of gliotoxin. Isolates were grown over 48 h in duplicate. At 48 h, fungal biomass was determined using an XTT reduction assay. Eight *A. niger* isolates and one *A. terreus* isolate were excluded due to insufficient growth by 48 h. OD, optical density.

positive control. The lower limit of quantitation of gliotoxin in culture media by this method was 25 ng/ml.

Preliminary experiments were performed in duplicate with a well-characterized isolate of *Aspergillus fumigatus* (AF 293) known to produce gliotoxin (5) to determine the optimal time point for gliotoxin sampling. Briefly, a standardized inoculum (2×10^5 conidia/ml) prepared in RPMI growth medium was transferred (10 ml) to replicate 15-ml vented polypropylene tubes and incubated with gentle shaking at 37°C for 96 h. At serial time points (0, 24, 48, 72, and 96 h), a tube was removed from the incubator, and culture medium was collected and stored at −80°C until analysis of gliotoxin concentration by HPLC. Fungal biomass was determined using the 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction assay described by Meletiadis et al. (6). In these preliminary studies, gliotoxin was detected as early as 24 h, and peak fungal biomass was reached at 48 h (data not shown). Gliotoxin concentrations in culture medium increased from a mean of 200 ng/ml at 48 h to approximately 1,500 ng/ml by 96 h. Therefore, screening culture medium at 48 h was sufficient for gliotoxin detection and allowed for optimal discrimination between isolates that produced minimal amounts of gliotoxin and those that produced gliotoxin heavily.

The growth rates of all 103 clinical *Aspergillus* isolates over 48 h were confirmed by XTT reduction assay prior to analysis of gliotoxin concentration in the culture medium (Fig. 1). Gliotoxin was frequently detected in the culture media of *A. fumigatus* and *A. niger* but detected less often in cultures of *A. terreus* and *A. flavus* (Fig. 2A). Similarly, mean concentrations of gliotoxin were significantly higher in *A. fumigatus* (628 ng/ml; range, 25 to 1,964) and *A. niger* (603 ng/ml; range, 51 to 1,093) cultures than in *A. terreus* (169 ng/ml; range, 49 to 466) and *A. flavus* (62 ng/ml) cultures (Fig. 2B). Review of patient medical records with stratification based upon EORTC/NIAID/MSG diagnostic criteria (1) revealed that 35/103 (34%) patients were likely colonized with *Aspergillus*, 48/103 (47%)

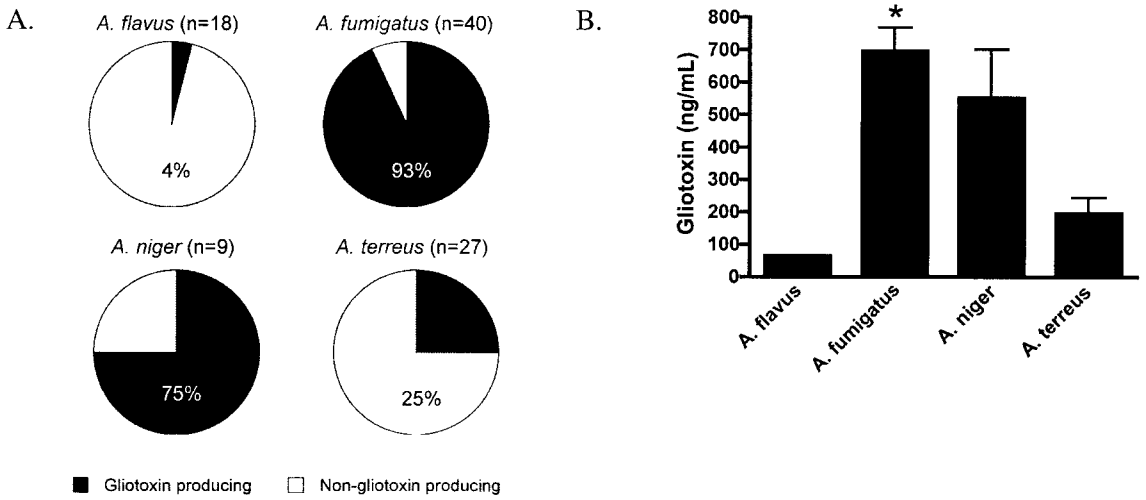

FIG. 2. Frequencies (A) and degrees (B) of gliotoxin production among clinical *Aspergillus* isolates. The bars in panel B represent mean concentrations with standard deviations. $*P < 0.01$ versus *A. terreus* and *A. flavus* by ANOVA with Tukey's posttest for multiple comparisons (Prism 4; Graphpad, San Diego, CA).

had probable infection, and 20/103 (19%) and definitive invasive aspergillosis. No differences in species distributions or in frequencies and degrees of gliotoxin production were noted across diagnostic stratifications. Specifically, the frequencies of gliotoxin production were similar for colonizing and invasive isolates recovered from high-risk patients (35 versus 46%, respectively). Additionally, no correlation between gliotoxin production and the severity of invasive aspergillosis, including in the dissemination of infection, was observed.

These data confirm that the ability to produce gliotoxin is discontinuously distributed among clinical *Aspergillus* isolates. While non-gliotoxin-producing isolates were recovered from patients with evidence of invasive infection, the most common species associated with invasive aspergillosis, *A. fumigatus*, was also the most consistent and abundant producer of gliotoxin among all of the species tested. Although the implications of gliotoxin production in vivo are not fully understood, the concentrations in culture media at 48 h were similar or surpassed concentrations reported to have immunosuppressive effects (25 to 200 ng/ml) on macrophages and mature lymphocytes (2, 8, 10, 14). An important limitation of our analysis is that we screened for a single mycotoxin only. Therefore, we cannot exclude the possibility that the non-gliotoxin-producing strains in our collection were producing alternative mycotoxins or other forms of gliotoxin. From a diagnostic standpoint, gliotoxin can be detected in the sera of patients with invasive aspergillosis (range, 166 to 785 ng/ml) (5), but data describing the frequencies or time courses of gliotoxin concentrations in the serum, respiratory secretions, or urine of patients infected with *A. fumigatus* versus those infected with non-*A. fumigatus* species are not yet available. Further studies to better define the role of gliotoxin in the pathogenesis of invasive aspergillosis, as well as the screening of patient samples for mycotoxins as an early diagnostic marker of invasive aspergillosis, are required.

REFERENCES

1. **Ascioglu, S., J. H. Rex, B. de Pauw, J. E. Bennett, J. Bille, F. Crokaert, D. W. Denning, J. P. Donnelly, J. E. Edwards, Z. Erjavec, D. Fiere, O. Lortholary, J. Maertens, J. F. Meis, T. F. Patterson, J. Ritter, D. Selleslag, P. M. Shah, D. A. Stevens, and T. J. Walsh.** 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin. Infect. Dis. **34:**7–14.
2. **Eichner, R. D., M. Al Salami, P. R. Wood, and A. Mullbacher.** 1986. The effect of gliotoxin upon macrophage function. Int. J. Immunopharmacol. **8:**789–797.
3. **Gardiner, D. M., P. Waring, and B. J. Howlett.** 2005. The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. Microbiology **151:**1021–1032.
4. **Latge, J. P.** 2001. The pathobiology of *Aspergillus fumigatus*. Trends Microbiol. **9:**382–389.
5. **Lewis, R. E., N. P. Wiederhold, J. Chi, X. Y. Han, K. V. Komanduri, D. P. Kontoyiannis, and R. A. Prince.** 2005. Detection of gliotoxin in experimental and human aspergillosis. Infect. Immun. **73:**635–637.
6. **Meletiadis, J., J. W. Mouton, J. F. Meis, B. A. Bouman, J. P. Donnelly, and P. E. Verweij.** 2001. Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. J. Clin. Microbiol. **39:**3402–3408.
7. **Mullbacher, A., P. Waring, and R. D. Eichner.** 1985. Identification of an agent in cultures of *Aspergillus fumigatus* displaying anti-phagocytic and immunomodulating activity in vitro. J. Gen. Microbiol. **131:**1251–1258.
8. **Murayama, T., R. Amitani, Y. Ikegami, R. Nawada, W. J. Lee, and F. Kuze.** 1996. Suppressive effects of *Aspergillus fumigatus* culture filtrates on human alveolar macrophages and polymorphonuclear leucocytes. Eur. Respir. J. **9:**293–300.
9. **Pahl, H. L., B. Krauss, K. Schulze-Osthoff, T. Decker, E. B. Traenckner, M. Vogt, C. Myers, T. Parks, P. Warring, A. Muhlbacher, A. P. Czernilofsky, and P. A. Baeuerle.** 1996. The immunosuppressive fungal metabolite gliotoxin specifically inhibits transcription factor NF-kappaB. J. Exp. Med. **183:** 1829–1840.
10. **Stanzani, M., E. Orciuolo, R. Lewis, D. P. Kontoyiannis, S. L. Martins, L. S. St John, and K. V. Komanduri.** 2005. *Aspergillus fumigatus* suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes. Blood **105:**2258–2265.
11. **Sutton, P., N. R. Newcombe, P. Waring, and A. Mullbacher.** 1994. In vivo immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi. Infect. Immun. **62:**1192–1198.
12. **Tomee, J. F., and H. F. Kauffman.** 2000. Putative virulence factors of *Aspergillus fumigatus*. Clin. Exp. Allergy **30:**476–484.
13. **Waring, P., R. D. Eichner, and A. Mullbacher.** 1988. The chemistry and biology of the immunomodulating agent gliotoxin and related epipolythiodioxopiperazines. Med. Res. Rev. **8:**499–524.
14. **Waring, P., R. D. Eichner, A. Mullbacher, and A. Sjaarda.** 1988. Gliotoxin induces apoptosis in macrophages unrelated to its antiphagocytic properties. J. Biol. Chem. **263:**18493–18499.